

CLAIMS

What is claimed is:

1. A method of producing a biologically active anti-angiogenic protein, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:
 - (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an anti-angiogenic protein, or a mutant, derivative, fragment or fusion protein thereof, into a yeast expression vector, wherein the vector contains a multiple cloning site; and
 - (b) transforming an appropriate yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the anti-angiogenic protein, thereby producing a biologically active anti-angiogenic protein, or mutant, derivative, fragment or fusion protein thereof.
2. The method of Claim 1 wherein the yeast strain is *Pichia pastoris*.
3. The method of Claim 1 wherein the expression vector comprises the pPICZ α A vector.
4. The method of Claim 1 wherein the biological activity is evaluated by one, or more of the following assays: endothelial cell migration; inhibition of tumor growth in a mammals; arrest of endothelial cells in G₁ phase of the cell cycle; or induction of apoptosis in endothelial cells.
5. The polypeptide encoding an anti-angiogenic protein wherein the anti-angiogenic protein, mutant, derivative, fragment or fusion protein thereof, wherein the protein is selected from the group consisting of: endostatin,

angiostatin or restin, or any mutants, derivatives, fragment or fusion protein thereof, or any combination thereof.

6. The method of Claim 1 wherein the protein, mutant, derivative, fragment or fusion protein is produced at a concentration of 10-20 milligrams or more per liter of culture fluid.
7. A biologically active anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced by the method of Claim 1.
8. The method of Claim 1 wherein the isolated polynucleotide of step (a) additionally comprises a polynucleotide linker and the anti-angiogenic protein, mutant, derivative, fragment or fusion protein thereof produced in step (b) additionally comprises at least one amino acid residue resulting from the linker polynucleotide.
9. The method of Claim 8 wherein the anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced comprises two additional amino-terminus amino acid residues.
10. The protein, mutant, derivative, fragment or fusion protein of Claim 9 wherein the amino acid residues are glutamic acid (E) and phenylalanine (F).
11. A biologically active anti-angiogenic protein, mutant, derivative fragment or fusion protein produced by the method of Claim 8.
12. The polypeptide of Claim 8 encoding an anti-angiogenic protein wherein the anti-angiogenic protein, or mutant, derivative, fragment or fusion protein thereof, wherein the protein, is selected from the group consisting of: endostatin, angiostatin or restin, or any combination thereof.

13. A composition comprising an anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced by the method of Claim 8 and a pharmaceutically acceptable carrier.
14. The method of Claim 1 wherein the vector of step (a) comprises a pPICz α A plasmid wherein the plasmid contains a multiple cloning site, said cloning site comprising a His.Tag motif and wherein the anti-angiogenic protein, mutant, derivative, fragment or fusion protein thereof produced in step (b) comprises a histidine tag motif.
15. The method of Claim 14 wherein the yeast strain is *Pichia pastoris*.
16. The method of Claim 14 wherein the biological activity is evaluated by one, or more of the following assays: endothelial cell migration; inhibition of tumor growth in a mammals; arrest of endothelial cells in G₁ phase of the cell cycle; or induction of apoptosis in endothelial cells.
17. The method of Claim 14 wherein the protein, mutant, derivative, fragment or fusion protein is produced at a concentration of 10-20 milligrams or more per liter of culture fluid.
18. A biologically active anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced by the method of Claim 14.
19. The polypeptide of Claim 14 encoding an anti-angiogenic protein wherein the anti-angiogenic protein, or mutant, derivative, fragment or fusion protein thereof, wherein the protein, is selected from the group consisting of: endostatin, angiostatin or restin, or any combination thereof.

20. A composition comprising an anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced by the method of Claim 1 and a pharmaceutically acceptable carrier.
- 5 21. A method of using an anti-angiogenic protein, mutant, derivative, fragment or fusion protein produced by the method of Claim 1 to inhibit undesirable angiogenesis in a mammal, comprising administering to the mammal an effective amount of the anti-angiogenic protein, mutant, derivative, fragment or fusion protein thereof.
- 10 22. A method of producing a biologically active anti-angiogenic protein, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:
- 15 (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an anti-angiogenic protein, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a pPICZ α A plasmid wherein the plasmid contains a multiple cloning site; and
- 20 (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the anti-angiogenic protein comprising at least one amino acid residue resulting from the linker polynucleotide, thereby producing a biologically active anti-angiogenic protein, or a mutant, derivative, fragment or fusion protein thereof.
- 25 23. The method of Claim 22 wherein the polynucleotide encodes angiostatin, endostatin, restin or mutants derivatives, fragments or fusion proteins thereof, or any combination thereof.

24. A biologically active anti-angiogenic protein produced by the method of Claim 22.

25. A method of producing a biologically active anti-angiogenic protein, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:

5 (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an anti-angiogenic protein, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a pPICz α A plasmid wherein the plasmid contains a multiple cloning site and wherein the cloning site additionally comprises a histidine tag motif; and

10 (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the anti-angiogenic protein comprising at least one amino acid residue resulting from the linker polynucleotide, and wherein the protein additionally comprises a histidine tag motif,

15 thereby producing a biologically active anti-angiogenic protein, or a mutant, derivative, fragment or fusion protein thereof.

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26. The method of Claim 25 wherein the polynucleotide encodes endostatin, angiostatin or restin, or mutants, derivatives, fragments or fusion proteins thereof, or any combination thereof.

27. A biologically active anti-angiogenic protein produced by the method of Claim 25.

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28. A method of producing biologically active angiostatin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:
- (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding angiostatin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a polynucleotide linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a pPICz α A plasmid wherein the plasmid contains a multiple cloning site; and
- (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the angiostatin comprising at least one amino acid residue resulting from the linker polynucleotide, thereby producing biologically active angiostatin, or mutant, derivative, fragment or fusion protein thereof.
29. Biologically active angiostatin produced by the method of Claim 28.
30. A method of producing biologically active angiostatin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:
- (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an angiostatin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a linker, wherein the linker encodes at least one amino acid, into a yeast expression vector comprising a pPICz α A plasmid wherein the plasmid contains a multiple cloning site and wherein the cloning site additionally comprises a histidine tag motif; and
- (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the angiostatin wherein the protein comprises at least one amino acid residue resulting from the linker polynucleotide and a histidine tag motif.

thereby producing a biologically active angiostatin, or mutant, derivative, fragment or fusion protein thereof.

31. Biologically active angiostatin produced by the method of Claim 30.

32. A method of producing biologically active endostatin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:

5 (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding a endostatin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a polynucleotide linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a

10 pPICz α A plasmid wherein the plasmid contains a multiple cloning site; and

(b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production

15 of the endostatin comprising at least one amino acid residue resulting from the linker polynucleotide,

thereby producing a biologically active endostatin, or mutant, derivative, fragment or fusion protein thereof.

33. Biologically active endostatin produced by the method of Claim 32.

20 34. A method of producing biologically active endostatin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:

(a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an endostatin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises

25 a polynucleotide linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a

pPICz α A plasmid wherein the plasmid contains a multiple cloning site and wherein the cloning site additionally comprises a histidine tag motif; and

- 5 (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the endostatin comprising at least one amino acid residue resulting from the linker polynucleotide, wherein the protein additionally comprises a histidine tag motif,

10 thereby producing biologically active endostatin, or mutant, derivative, fragment or fusion protein thereof.

35. Biologically active endostatin produced by the method of Claim 34.

36. A method of producing biologically active restin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:

- 15 (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an restin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a linker, wherein the polynucleotide linker encodes at least one amino acid, into a yeast expression vector comprising a pPICz α A plasmid wherein the plasmid contains a multiple cloning site; and

- 20 (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the restin comprising at least one amino acid residue resulting from the linker polynucleotide,

25 thereby producing a biologically active restin, or mutant, derivative, fragment or fusion protein thereof.

37. Biologically active restin produced by the method of Claim 36.

38. A method of producing biologically active restin, or a biologically active mutant, fragment, derivative or fusion protein thereof, comprising:
- 5 (a) inserting an isolated polynucleotide comprising a polynucleotide sequence encoding an restin, or a mutant, derivative, fragment or fusion protein thereof, wherein the polynucleotide additionally comprises a linker, wherein the linker encodes at least one amino acid, into a yeast expression vector comprising a pPICZ α A plasmid wherein the plasmid contains a multiple cloning site and wherein the cloning site additionally comprises a histidine tag motif; and
- 10 (b) transforming a *Pichia pastoris* yeast strain with the vector of step (a) and maintaining the yeast strain under suitable conditions for the production of the restin wherein the protein comprises at least one amino acid residue resulting from the linker polynucleotide and a histidine tag motif.
- 15 thereby producing a biologically active restin, or mutant, derivative, fragment or fusion protein thereof.
39. Biologically active restin produced by the method of Claim 38.